THE INFLUENCE OF THE H-ION CONCENTRATION ON THE GROWTH OF B. TYPHOSUS IN MEDIUMS CONTAINING BILE OR BILE SALTS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VIII.

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In the preceding paper it was demonstrated that bile, particularly hepatic duct specimens, acquired germicidal properties when exposed to air. Comparative tests indicated that this effect was connected with a lowering of the H-ion concentration. However, it was not entirely evident in what manner this change in the reaction, alone or in combination with the chemical elements of the bile, had acted as the germicidal factor. A few experiments with bile specimens and bile salts at varying H-ion concentrations suggested themselves. These tests, primarily undertaken to study the rate of growth of B. typhosus and its generation time, furnished data which indicated that a low H-ion concentration inhibited the growth-stimulating properties of the bile and its salts and rendered such a medium germicidal.

With the exception of Meyerstein,[1] who states that the growth of B. coli and B. typhosus is abundant only when the mediums containing bile salts are neutral or slightly acid to litmus, recent reports on the subject of ox bile as a culture medium fail to mention the importance of the reaction. Ecker [2] and Salter [3] use neutral or slightly acid mediums which, according to the findings of Meyerstein, are the optimum reaction. Such a favorable reaction is, according to our tests, rarely encountered with the usual ox bile samples obtained in a fresh state from abattoirs, and sterilized while warm. It is quite obvious that the reports of various investigators dealing with the nutritive value of bile for bacteria must be more or less at variance on account of their failure to adjust the reaction of bile or bile salt mediums. Moreover, the proper-

Received for publication Dec. 31, 1920.

[1] Centralbl. f. Bakteriol., O., I, 1907, 44, p. 434.
[2] Jour. Infect. Dis., 1918, 22, p. 95.
[3] Ibid., 1919, 24, p. 260.

ties of sodium taurocholate or glycocholate and their decomposition products are not accurately known. On the other hand, it is not our intention to overemphasize the reaction of the bile salt mediums, but in connection with the experimental pathologic studies reported in the preceding papers, it appeared to us as one of the important factors among the many dealing with the so-called antiseptic effect of bile. In order to obtain striking contrasts, two reactions, namely, P_H 7.0 and P_H 8.2 to P_H 8.4 were chosen.

Technic.—One strain of B. typhosus (K) was used throughout the entire series of experiments. Transplants were kept on peptic digest agar. Previous to an experiment it was transferred for a few days to 1% ox bile salt-free veal broth. For each test a 18-24 hour old culture in this medium was used.

The salt-free veal broth was prepared in the same manner as stated in the second paper of this series. The 0.01% peptone solution, which was suggested by the work of Meyerstein, was made by dissolving 0.02% "Difco" peptone in distilled water and adding 20 c c of a phosphate mixture of known H-ion concentration to each 100 c c of medium. This mixture was distributed in 30 c c amounts in Pyrex Erlenmeyer flasks; sterile, filtered or unfiltered bile, or any other bile product to be studied was added and the total volume was made up to 50 c c. Sterilization was carried out in live steam on three consecutive days.

A 0.01% peptone solution, according to Meyerstein, is supposed to maintain the life of the organisms and yet not furnish enough nutritive material to permit a vigorous growth of B. typhosus. Hence any inhibitive or stimulating properties possessed by bile samples or their salts would be definitely demonstrated.

Several fresh samples of ox bile (12-14 cystic bile specimens) were filtered through paper and sterilized at 15 pounds' pressure. Sterilly collected hepatic duct bile samples of several rabbits were pooled and added without sterilization to the medium. Some experiments were also carried out with bile derived from a single gallbladder of the ox. Desiccated, "Difco" ox-bile was dissolved in distilled water and the concentrated solution added to the basic nutritive solution. This preparation is only slightly soluble in distilled water; a 1% solution forms an appreciable precipitate in the neutral medium. The latter was not filtered from the test medium, but care was taken to rotate the flasks thoroughly to insure even distribution of the sediment before samples for plating were removed. The purified bile salts were prepared by Drs. Foster and Hooper from dogs' or pigs' bile and have been tested by them in their studies on the metabolism of bile acids.

Fifty c c of medium were seeded with 0.5 c c of a 1:10,000 dilution of a young culture of B. typhosus in 1% ox bile salt-free veal broth. The flasks were warmed to 37 C. before inoculation and kept incubated at this temperature in an electrically controlled water bath. The determination of the number of organisms present at various periods of growth were made by plating in peptic digest agar (P_H 7.0). This medium was prepared in large quantities to insure uniformity in composition. Duplicate and often triplicate plates were poured with dilutions made in sterile salt solution. The plates were counted after 48

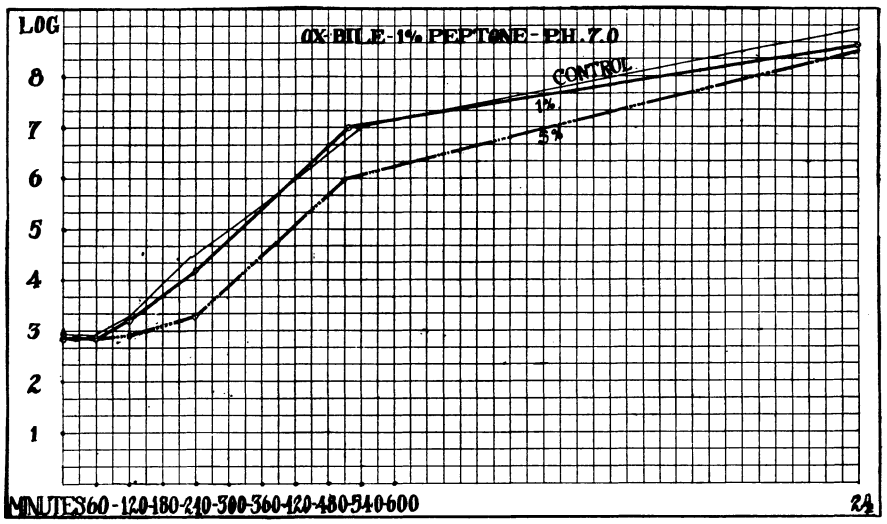

Chart 1.—Rate of growth of B. typhosus in filtered sterile ox bile in 1% peptone-phosphate solution, PH 7.0.

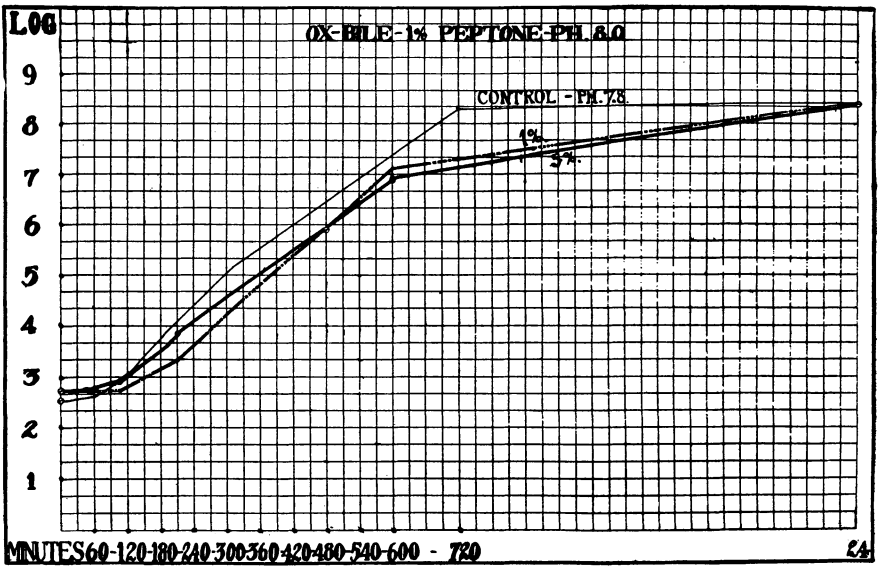

Chart 2.—Rate of growth of B. typhosus in filtered sterile ox bile in 1% peptone-phosphate solution, PH 8.0.

hours' incubation at 37 C. Only plates which showed more than 25 or less than 250 colonies were considered accurate. Each experiment was repeated, and only the results in which no marked changes in the P_H reaction of the test medium occurred were noted. Representative data of such series are chosen for discussion of the individual experiments.

In the charts the logarithms of the viable organisms per 1 c c of medium are plotted as ordinates against time intervals (expressed in minutes) as abscissae.

Exper. 1.—Filtered sterile ox bile in 1% peptone-phosphate solution was seeded with B. typhosus and kept in water bath at 37 C.

This experiment clearly demonstrates that ox bile in low concentrations, namely 1 and 5% solutions, added to a suitable nutritive substratum, is distinctly inhibitive for B. typhosus. These observations confirm those recently published by Ecker. The growth curves of a 1% solution run below those of the control mediums throughout the entire period of the experiment. A 5% solution is decidedly depressant on the development of B. typhosus, a fact which is indicated by a distinct lag extending over 80 minutes. The final growth is, however, not materially influenced; the bile-curves show a tendency to reach the same level as the control-curve. Furthermore, the reaction of the medium does not materially alter the rate of growth, as is evidenced from the general behavior of the two curves. The minor differences in the character of the curves may be safely attributed to technical irregularities.

It was quite obvious that a 1% peptone solution was unsuitable to demonstrate conclusively the inhibitive or even the germicidal properties of bile. The available nutritive substances permitted a good initial growth, and the subsequent adaptation to the antagonistic forces of the bile produced the results clearly demonstrated in the curves. Identical observations were made by Pies.[4] The suggestion of Meyerstein to use a 0.01% peptone solution was therefore followed. It was unfortunate that our experiments, which were intended to retain the initial reaction constant, necessitated the addition of a phosphate mixture. A peptone-phosphate solution is, in contrast to Meyerstein's Witte's peptone solution, a fair culture medium, as the figures in the table clearly demonstrate.

It is evident from the table that the claims of Meyerstein were not confirmed. In several tests a distinct increase of the inoculated B. typhosus took place after 24 hours' incubation. The multiplication

[4] Arch. f. Hyg., 1907, 62, p. 107.

was not as marked as in a "Difco" peptone solution, or in the same solution buffered with phosphates. The phosphate mixture at a P_H 7.0, kept B. typhosus alive or stimulated a slight growth. It is, therefore, advisable to test in such a solution the substances which are suspected to exert an inhibitive or germicidal effect on bacteria. For the graphic demonstration of the influence of the reaction on the action of bile salts a 0.01% "Difco" peptone solution with phosphates reproduced the nutritive value of the bile more accurately than the phosphate mixture alone. This conclusion was justified by the numerous successful tests to be reported.

GROWTH OF B. TYPHOSUS IN PEPTONE AND PEPTONE-PHOSPHATE MIXTURES PH 7.0

	Number of Organisms Immediately After Inoculation per C c	Number of Organisms After 24 Hours per C c
Phosphate mixture alone	410	860
Difco peptone, 0.01% solution	1,600	13,000,000
Difco peptone, 0.01% solution + phosphate mixture	1,600	34,000,000
Witte's peptone, 0.01% solution	1,600	1,350,000
Witte's peptone, 0.01% solution + phosphate mixture	1,680	1,560,000

Exper. 2.—Filtered sterile ox bile in a 0.01 "Difco" peptone-phosphate solution, P_H 7.0 and P_H 8.2, was seeded with B. typhosus. Incubation occurred at 37 C. Four tests were made.

At a reaction of P_H 7.0, ox bile in quantities of 1% or less was decidedly stimulating for B. typhosus. Even the usual lag was entirely absent, and the figures obtained for the final growth exceeded the one for the control flask. Five, 10 and 30% additions of ox bile were not only inhibitive, as indicated by the prolonged flag, but were also depressing the growth curve in general. Attention is called to the inhibition of the growth for at least 5 hours in the 10 and 30% ox bile-peptone solutions. Moreover, in contrast to the 1% peptone solution, the development of B. typhosus in a 0.01% peptone medium was slow but progressive, and the curve failed to show the steep rise in the first 8 hours so clearly noted on charts 1 and 2.

At a P_H 8.2, ox bile, even in concentrations below 1%, was distinctly inhibitive. The initial lag for 10 and 30% solutions was extended over a period of from 8 to 9 hours. Furthermore, the final growth as a whole was considerably below the one noted for the medium of a P_H 7.0. In many respects the general character of the 30% ox bile curve corresponded to the one established in the preceding paper (chart 8) for fresh unsterilized ox bile of a reaction of P_H 8.2-8.6. These findings

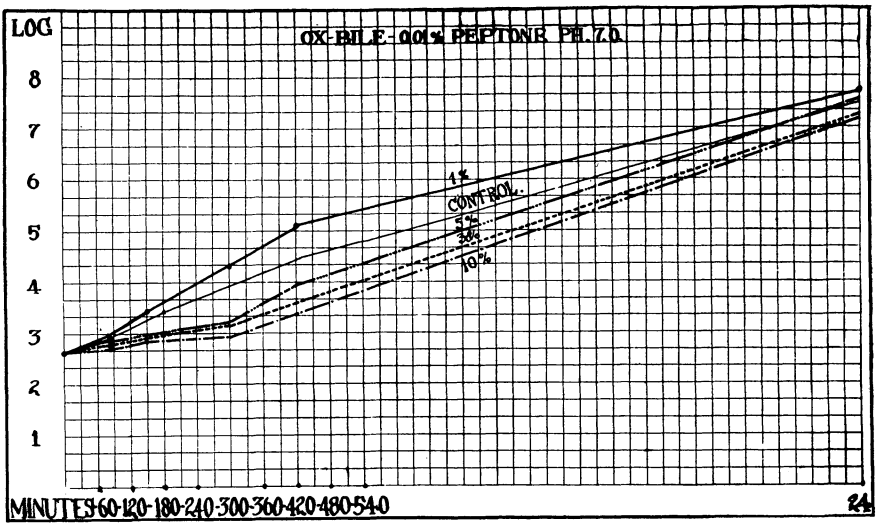

Chart 3.—Rate of growth of B. typhosus in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 7.0.

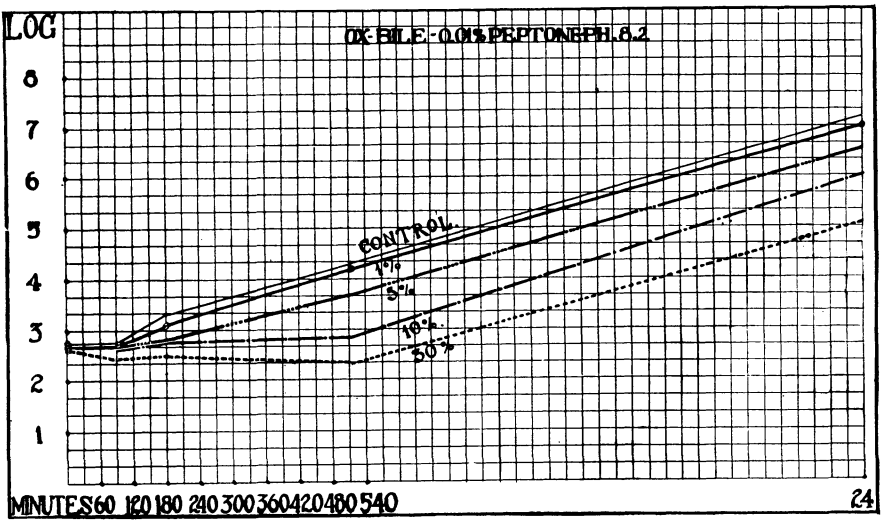

Chart 4.—Rate of growth of B. typhosus in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 8.2.

demonstrated that ox bile at a P_H 8.2 inhibits a large proportion of the viable cells.

The nature of the inhibitive substances in ox bile is for the present omitted from consideration. Whether the specific stimulating effect of this secretion in small concentrations at a P_H 7.0 is merely the result of additional nutritive material or due to certain definite substances, is difficult to say. It is recalled that Salter found bile salts in small quantities to be growth promoting for B. coli. Bile in low concentrations can therefore act in a similar manner on all members of the colon typhoid-paratyphoid group.

Exper. 3.—"Bacto" desiccated ox bile in 0.01% "Difco" peptone-phosphate solution, P_H 7.0 and 8.2, was seeded with B. typhosus. Incubation ocurred at 37 C. Two experiments were made.

The influence of the reaction on the effect of bile salts as culture mediums for B. typhosus was well illustrated by this experiment. Thus it was shown that neutrality supported the growth producing properties of a low concentration of bile salts, while a strongly alkaline reaction rendered the same substances not only inhibitive, but germicidal. At P_H 7.0 a 30% "bacto"-bile salt medium was slightly inhibitive and a 0.5 and a 1% solution were stimulative. The curve for a 2% bile salt medium was not shown in the chart. It covered the one for the control medium.

At a P_H of 8.2 a 1%, even a 0.2%, solution of desiccated bile was germicidal for B. typhosus. The rate of lethal action was shortened by the concentration of the medium in biliary elements or, in other words, the more concentrated the fluid, the greater was its effect on the viable cells. It was furthermore shown that the germicidal action did not manifest itself for at least 3 hours in the 0.2 and 0.5% bile salt medium. There were indications that for this time period the inoculated B. typhosus not only remained viable, but proliferated to a slight degree. This particular phenomenon was not absolutely constant and certain unknown factors, possibly similar to those noted by Cohen and Clark, produced slight variations. The curves shown in charts 5 and 6 represent the general behavior of B. typhosus in bile salt mediums at different H-ion concentrations, and find their analogues in some of the charts made from determinations on fresh, undiluted animal bile, discussed in the preceding paper.

Exper. 4.—Sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution was seeded and incubated as usual.

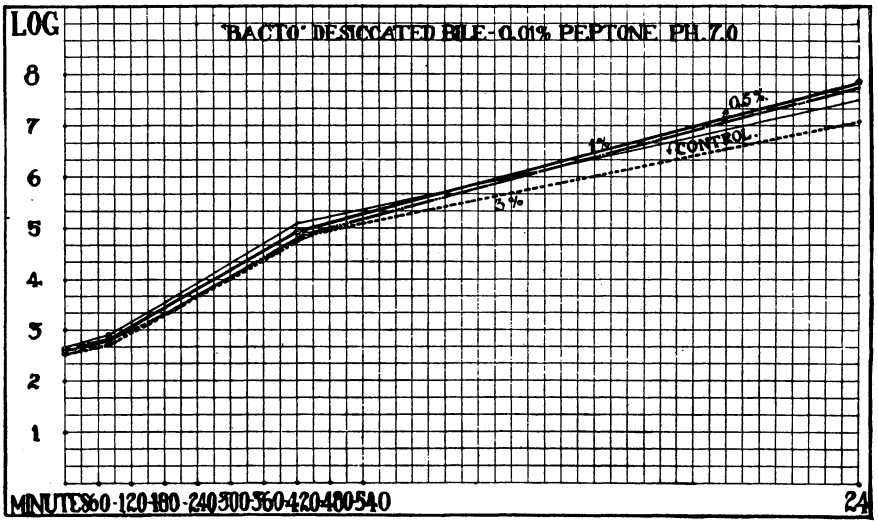

Chart 5.—Rate of growth of B. typhosus in "bacto" desiccated ox bile peptone-phosphate solution, PH 7.0.

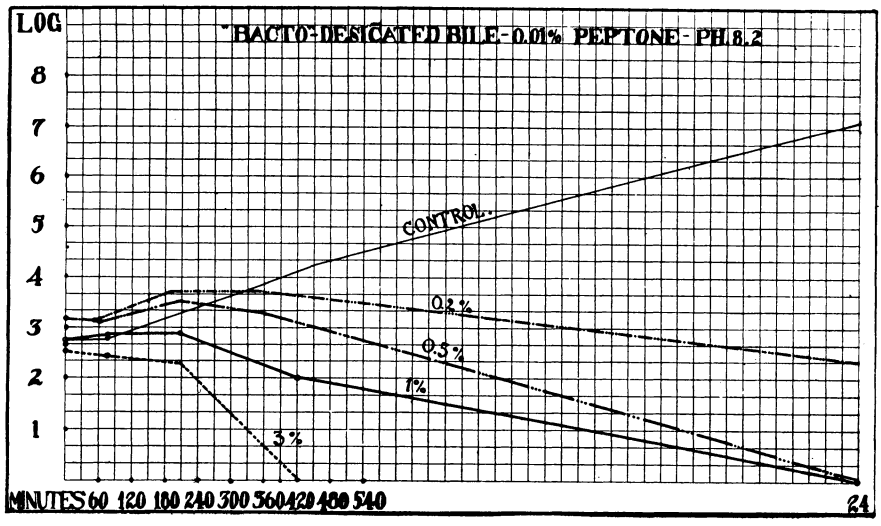

Chart 6.—Rate of growth of B. typhosus in "bacto" desiccated ox bile peptone-phosphate solution, PH 8.2.

The curves presented in charts 7 and 8 confirmed our previous observations with undiluted hepatic duct bile of the rabbit exposed to air for several days. However, the data revealed some interesting properties which were not evident in the tests conducted with undiluted bile. Rabbit bile was not only less inhibitive than ox bile in a neutral medium, but must be considered stimulating and growth enhancing. Additions of bile (not exceeding 10%) invariably produced better development of B. typhosus than a peptone-phosphate mixture alone. The paradoxical behavior of the 5 and 10% solutions of rabbit bile was constant in the 2 experiments conducted. For the present we are unable to offer an explanation for the observations, but desire to call attention to a similar observation recorded by Meyerstein. In the course of some tests with purified crystallized ox bile, this worker noted that B. typhosus and B. coli grew well in a 10% solution of a mixture of sodium taurocholate and sodium glycocholate (40 and 60%), while lower concentrations (1 to 5%) completely inhibited proliferation of these organisms. He states that this action of the bile salts was not recorded with the desired regularity to permit a final conclusion. In the light of the experiments conducted with pure bile salts, it is suspected that these paradoxic results are due to the glycocholic acid or its salts. Further tests are, however, necessary to prove this contention in a conclusive manner.

The general tendency of the growth curve at P_H 7.0 also indicated that the typhoid bacillus adapted itself more readily to the elements contained in the rabbit bile than to those of ox bile. The initial lag was comparatively short and between the 2nd and 6th hour the proliferation was very active in the 5 and 10% solutions. The final growth at the end of 24 hours, however, did not materially exceed the one reported for ox bile.

An alkaline reaction of P_H 8.4 affected the inoculated bacteria in a manner already known. In the 2 experiments the results differed somewhat from each other, but the curves shown in chart 8 present the average behavior of B. typhosus in rabbit bile at P_H 8.4. Moreover, the 5% solution acted paradoxically, while the 1 and 10% mediums followed the course to be anticipated. Both the 5 and 10% bile-peptone solutions exhibited distinct germicidal properties in the initial 5 hours, which was followed in the 5% solution by a slow, but progressive proliferation of the remaining viable cells. On the other hand, the 10% solution continued to destroy the inoculated germs until com-

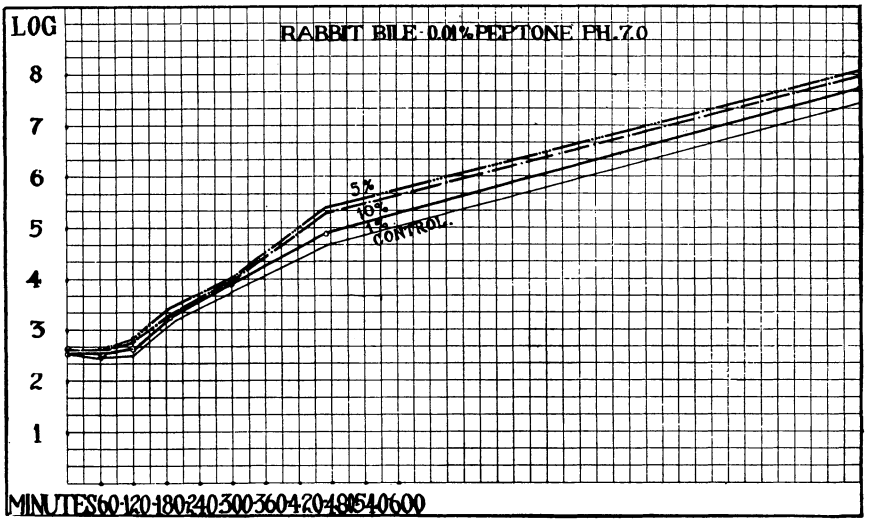

Chart 7.—Rate of Growth of B. typhosus in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 7.0.

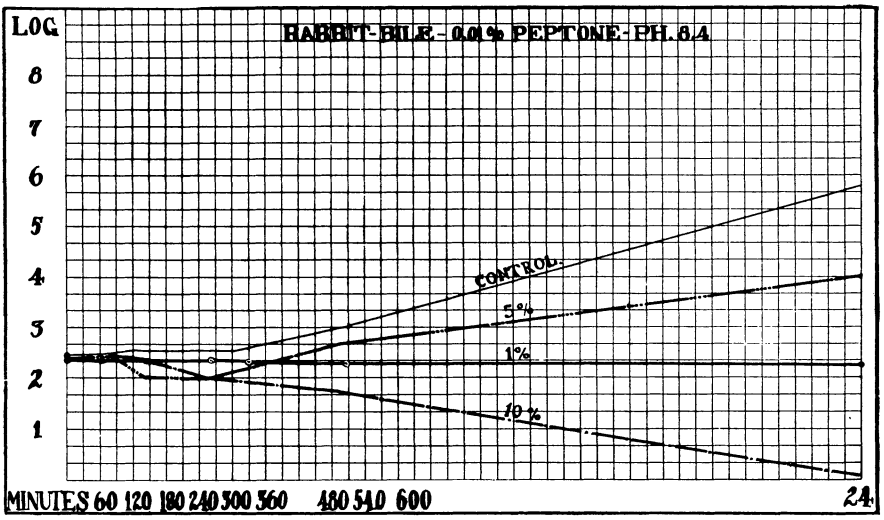

Chart 8.—Rate of growth of B. typhosus in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 8.4.

plete sterilization resulted in the 24th hour. The 1% solution exhibited a distinct bacteriostatic action. The available figures indicate that typhoid bacilli either remained alive or were slightly reduced in numbers. At P_H 8.4 rabbit hepatic duct bile added in varying amounts to peptone-phosphate solutions may therefore exhibit three different properties: it maintains growth, it is bacteriostatic, or it is germicidal. The various properties ascribed to animal bile by numerous investigators can obviously be recorded on one and the same specimen. The nature of the inhibitive substances has been considered in the preceding paper. It was concluded that in all probability the bile salts deserve a more detailed investigation. With this conception in mind a series of tests were conducted with pure bile salts.

Exper. 5.—Sodium taurocholate and sodium glycocholate in a 0.01% peptone-phosphate solution were seeded and incubated as usual. Three experiments were made.

A superficial inspection of the curves revealed the same tendency in the rate of growth of B. typhosus in pure bile salt mediums as already noted and discussed for the fresh or the desiccated ox bile. The growth of B. typhosus was excellent at P_H 7.0, but it was inhibited at P_H 8.4. Moreover, distinct differences existed between the peptone solution containing sodium glycocholate and those prepared with sodium taurocholate. At neutrality sodium glycocholate in 0.5% and 1% solutions was bacteriostatic for 3 hours, but at the end of 24 hours the number of viable cells exceeded those of the control flask. A somewhat similar effect was noted for the taurocholates. From the available data, however, it was impossible to state whether sodium glycocholate was more stimulative at P_H 7.0 than the sodium taurocholate. In comparison with the results obtained at a low H-ion concentration the observation deserves recognition and will repay additional investigation.

The outstanding feature of the action of purified bile salts at P_H 8.2 was the strongly germicidal effect of sodium glycocholate in 0.5% concentration, the inhibitive and slightly antiseptic effect of 1% sodium taurocholate, and the growth-depressing properties of the same salt in 0.5% solutions. It was quite evident that in alkaline mediums glycocholates were considerably more germicidal than the taurocholates, a fact which has hitherto not been emphasized. Meyerstein [5] noted the absence of growth of B. typhosus in a 0.01% Witte's peptone solution

[5] Centrlbl. f. Bakteriol., I, O., 1904, 44, p. 138.

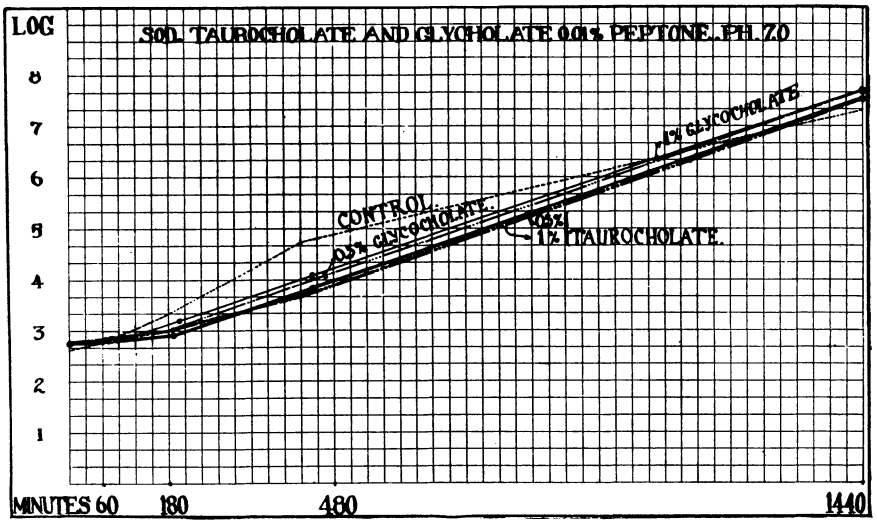

Chart 9.—Rate of growth of B. typhosus in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, PH 7.0.

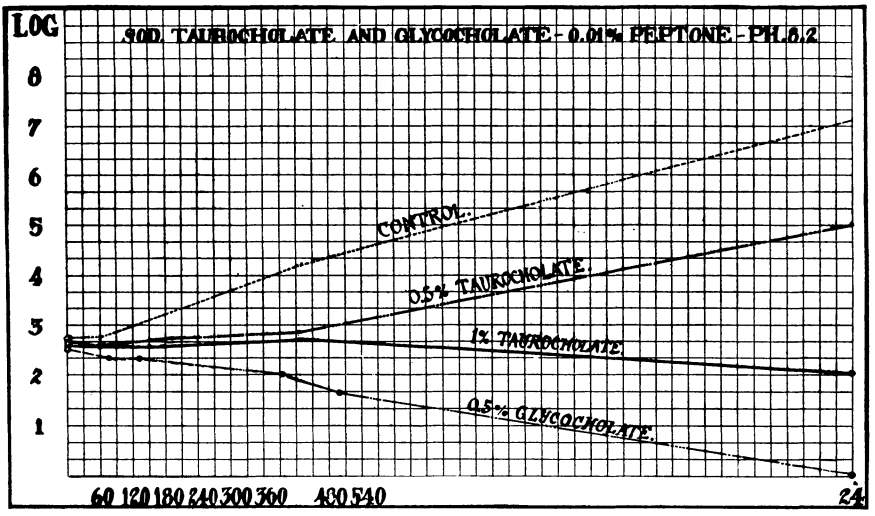

Chart 10.—Rate of growth of B. typhosus in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, PH 8.2.

containing 5% sodium glycocholate, while Dünschman [6] apparently made a similar observation, which prompted him to write the following sentence: "le glycocholate, de son côté agit plutôt à la façon d'un antiseptique."

Even the growth maintaining 0.5% sodium taurocholate-peptone solution was not indifferent to the typhoid bacillus: an 8-hour lag precedes the slow but progressive growth.

DISCUSSION

It is evident from the foregoing data that the rate of growth and the action of the bile and the bile salts of various animals on B. typhosus in simple mediums is materially influenced by the reaction. At neutrality small amounts of ox bile, "Bacto" desiccated bile, sodium taurocholate and glycocholates and fairly large amounts of hepatic duct bile of the rabbit are distinctly stimulating. These results are definitely demonstrable when a simple 0.01% peptone-phosphate solution is used as a basic substratum. As a rule, bile concentrations of over 1% are distinctly inhibitive at a P_H 7.0. Rabbit bile in this respect offers an exception for the following reason: It is technically impossible to obtain a sufficient amount of cystic bile to conduct a well-controlled series of tests. The hepatic duct bile employed is less concentrated and contains about $\frac{1}{10}$ of the organic and inorganic elements ordinarily encountered in the gallbladder bile of cattle. It is also possible that rabbit bile is an exceptionally good medium for B. typhosus at a neutral or slightly alkaline reaction. Our observations made in vivo and in vitro with cystic bile of these animals and reported in the preceding papers lend some support to this contention.

The nature of the stimulating action has not been investigated. It may be that the bile or bile salts supply only additional food material to a comparatively poor nutritive substratum. The bile and bile salts may contain some of the mysterious substances classified with the so-called "vitamines" that play an important rôle in animal metabolism. Viewed from this standpoint the bacteriologic aspect of bile offers an unlimited number of experimental possibilities.

In an alkaline medium (P_H 8.2 to 8.4) the bile specimens or their salts invariably cause distinct inhibition in concentrations below 0.5%. The addition of comparatively large amounts of the same bile or biliary salts renders the medium not only inhibitive, but directly germicidal.

[6] Ann. de l'Inst. Pasteur, 1909, 23, p. 48.

In all such mediums the lag is increased, and there may be a distinct bacteriostatic effect for from 5 to 10 hours. A slight initial growth may be followed by a slow, but progressive, destruction of the viable cells. In ox bile the prolonged lag may be replaced by an active growth leading after 24 hours to a number of viable cells nearly as great as the one recorded in the bile-free control tube. This phenomenon is probably the result of adaptation of B. typhosus to the antagonistic forces of the alkaline-bile mediums. It is possible that the inhibitive substances, as for example the bile salts, are decomposed as a result of the growth of B. typhosus (Exner and Heyrovsky) and transformed into growth-enhancing food elements. Our experiments are not sufficiently numerous, nor have they been planned with this question in mind. The data at our disposal permit one conclusion, namely, that the bile salts, and particularly the glycocholates, are the substances which inhibit or diminish the viable cells of B. typhosus in weak peptone solutions. Furthermore, the interesting and noteworthy fact is recorded that the bile salts in the concentration usually present in normal bile exert their germicidal properties when the medium is alkaline. In searching for an analogous condition, the result of studies of several investigators are recalled, namely, certain dyestuffs are more potent in a medium with a reaction in the alkaline range than in one at or near neutrality. Prowazeck [7] and Traube [8] found that sodium carbonate accentuates the toxicity of methylene blue or of crystal violet. Browning, Gulbranson and Kennway [9] also obtained remarkable results in the sterilizing effect of diaminoacricidine-methyl-chloride by changing the P_H of the medium from 4.0 to 11.0. Neither of these workers offers a conclusive explanation for this phenomenon, but it is not unlikely that the view of Traube, who found that sodium carbonate produced changes in the surface tension of the dye solutions, is applicable to the bile salts mediums. In this connection the observations of Larson, Cantwell and Hartzell [10] with pneumococci should be mentioned. Bile reduces the surface tension of fluids and, according to the foregoing writers, favors the disintegration of pneumococci. How far our findings are analogous to those just stated must be determined by further tests. One fact is certain: Bile salts are more readily soluble in alkaline solution than in neutral or slightly acid mediums.

[7] Arch. f. Protist., 1910, 18, p. 221.
[8] Biochem. Ztschr., 1912, 43, p. 496.
[9] Jour. Path. & Bacteriol., 1919, 23, p. 106.
[10] Jour. Infect. Dis., 1919, 25, p. 45.

For the present the selective germicidal action of glycocholates in alkaline mediums cannot be explained. An observation of considerable practical importance is that bile and bile salts derived from various animals contain varying amounts of this acid. According to Hammarsten,[11] rabbit bile and some samples of ox bile possess exclusively glycocholates; while dog bile is stated to be deprived of this substance. Variation in the cultural properties of bile samples must be ascribed to the composition of the secretion, and not purely to the reaction, as is the case in the observations of Nichols. Chemical analyses of hepatic duct bile of rabbits are not available for comparison, but it is not unlikely that similar unexplicable fluctuation in the bile acid content may occur, as has been so clearly demonstrated by Foster, Hooper and Whipple [12] for the dog. Future bacteriologic studies on bile should therefore appreciate not only the variability of the reaction, but of the composition as well.

In all probability the antiseptic action of glycocholates or taurocholates does not occur in the animal body. A reaction conducive to the development of germicidal properties is found only in the test tube exposed to air, and not in the gallbladder in vivo. This point has been treated in detail in the preceding papers.

As far as the recorded observations have some practical bearing on the use of bile or bile salt mediums, it can be stated that such additions to nutritive mediums will be advantageous only in neutral or slightly acid substratums, and when the concentration does not exceed 1% fresh or 0.5-1% desiccated ox bile. The value of bile additions should never be over-estimated, primarily on account of the comparatively slight stimulating, selective effect on B. typhosus, secondarily on account of the production of a distinct lag, which is provoked by fairly small amounts of bile salts. The recommendation of Tonney, Caldwell and Griffin [13] in disregarding lactose bile for the isolation of B. typhosus from the stool is fully justified in the light of our findings.

CONCLUSIONS

Bile of oxen, hepatic duct bile of rabbits, bacto "desiccated ox bile," sodium glycocholate and taurocholate in 1% concentration in a 0.01% "Difco" peptone-phosphate solution at a P_H 7.0 are growth-enhancing

[11] Ergebn. d. Physiologie, 1905, 4, p. 1.
[12] Jour. Biol. Chem., 1919, 38, p. 379.
[13] Jour. Infect. Dis., 1916, 18, p. 239.

for B. typhosus, while greater amounts, such as 3 to 30%, greatly inhibit proliferation. At P_H 8.4 the same bile specimens or their salts acquire either inhibitive, bacteriostatic or germicidal properties. The more concentrated the mediums are in biliary salts, the greater is their effect on the viable cells. Even small amounts of bile salts, such as 0.5%, destroy the inoculated bacteria in 24 hours. At P_H 8.4 glycocholates are more antiseptic than taurocholates, while the same salts in the same concentration may be stimulative at a P_H 7.0.